Optimization of an in situ forming implant system for long-acting Human Immunodeficiency Virus (HIV) Prevention

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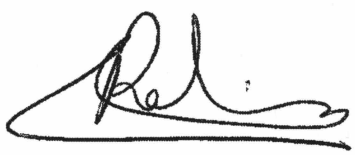

Abstract

Introduction:
The only pre-exposure prophylaxis (PrEP) method on the market, once daily oral pill Truvada, demonstrates variable efficacy that is largely correlated to patient adherence. Long-acting injectables (LAI's) delivering various antiretrovirals (ARVs) are a promising approach to circumvent the challenges presented by Truvada and other PrEP methods. Dolutegravir (DTG) was successfully formulated, in previous work, in an in-situ forming implant (ISFI) delivery-system containing 50:50 poly(DL-lactide-*co*-glycolide) (PLGA) and *N*-methyl-2-pyrrolidone (NMP). This formulation produced mean plasma concentrations of DTG that were close, yet did not surpass four times the concentration needed to inhibit 90% of viral replication (IC90) in a rhesus macaque model – the understood efficacy threshold of PrEP methods. The aim of this present work was to develop and characterize an ISFI formulation with increased dolutegravir loading to reach mean plasma concentrations greater than 4x the IC90 of dolutegravir. The overall goal was to optimize a formulation that would achieve a concentration of 300mg/mL (for a target human dose of 600 mg DTG in 2mL) for a single injection that provides HIV-1 protection for at least three months.

Materials and Methods:
The solubility of DTG was evaluated in various organic solvents and solvent ratios. DTG was then formulated within the ISFI with the selected optimized system, NMP: Gelucire® 44/14 (9:1) and was further evaluated for efficacy and safety in *in vitro* and *in vivo* models. In addition, density, viscosity, and stability of this formulation was evaluated. Scanning electron microscopy (SEM) and differential scanning calorimetry (DSC) were also used to evaluate the physical characteristics of the formulation.

Results and Conclusion:
The optimized ISFI formulation was comprised of 50:50 PLGA, NMP, Gelucire and DTG. In-vitro release studies indicated that PLGA: (NMP/Gel 9:1) formulation ratio of 1:4 gave the favored release profile to translate into a desired release profile in vivo, total cumulative drug released being 57.53% at 121 days. Safety results indicate that the optimized formulation was very well tolerated,as there was little to no inflammation indicated by little to no macrophage/lymphocyte presence and no detectable pro-inflammatory cytokines (INF-α or IL-6). An In-vivo pharmacokinetic study evaluated the 1:2 and 1:4 formulation ratios. Compared to a 1:2 formulation ratio containing NMP alone, the 1:2 and 1:4 PLGA: (NMP/Gel 9:1) formulation ratios were found to increase mean plasma concentrations 3.86-fold and 5.55-fold at 30 days post-injection. This ISFI formulation with an optimized solvent system demonstrated improved DTG loading capacity and pharmacokinetic results, in vivo, and thus support its further development as a more appropriate alternative to current long-acting PrEP methods in development.

1. Introduction

Human Immunodeficiency Virus (HIV) remains a global health crisis, and despite a dramatic decrease in Acquired Immune Deficiency Syndrome (AIDS) related deaths worldwide, there remains serious room for improvement for both HIV treatment and prevention.[1,2] Strides have been made with the use of antiretrovirals (ARVs), in pre-exposure prophylaxis (PrEP), administered to those at highest risk, notably with the recent the Food and Drug Administration (FDA) approval of a once daily oral pill, Truvada (tenoforvir disoproxil fumarate + emtricitabine) in 2012. The efficacy of Truvada varies from prevention of transmission in zero to 75% of cases, and prevention is largely correlated to patient adherence. Only about 50-80% of patients had detectable plasma levels of tenoforvir disoproxil fumarate at measured time points, a surrogate measure of adherence. Concerns regarding patient adherence, and development of drug resistance, have been raised across health care disciplines.[3-6] A phase III clinical trial demonstrated no efficacy of a tenofovir 1% vaginal gel used before or immediately post-coitus compared to placebo, in an attempt to circumvent varied adherence among patients.[5] Current literature presents long-acting PrEP as a promising approach to the demand of improved efficacy and adherence in HIV prophylaxis.[8]

Sustained-release intravaginal rings have been championed in the development of long acting PrEP. MTM-020-ASPIRE and IMP-027 clinical trials presented a 27% and 30.7% reduction in HIV infection respectively in subjects using the ring versus placebo. With these prevention rates, subgroup analyses showed that the greater adherence group experienced greater efficacy. This further illustrated the importance of patient adherence in the success of prevention methods. [9-11] Efforts are now focused on long acting injectables (LAIs) to overcome the challenges of intravaginal rings such as patient adherence, tactile sensation of the ring during sexual intercourse, and absence of rectal protection from infection.[9,10] LAI's have already demonstrated substantial success in the formulation of antipsychotic, contraception, diabetic and opioid-dependence agents.[12-15]

While there are no FDA approved LAIs for HIV prophylaxis on the market currently, there are two LAIs in clinical development. Notably, a phase II study of GSK 744-LA, a cabotegravir (CAB, formerly GSK 1265744, ViiV Healthcare) loaded system was recently completed and a phase III and phase II/III have since been initiated and are currently ongoing.[16-19] A phase IIb study using both GSK 744-LA and TMC 278-LA, a rilpivirine (RPV, Edurant, Janssen Scientific) loaded system, together versus oral agents has also been initiated. LAI systems in clinical development are crystalline nanosuspensions for intramuscular (IM) injection.[20,21] Elan's Nanocrystal™ technology is utilized to achieve a stable formulation with high drug loading capacity. Cabotegravir or rilpivirine are milled to a median particle size of 200 nm and suspended in an aqueous vehicle containing surfactants, along with a polymer and tonicity agent in the GSK 744-LA/GSK 1265744 formulation. Both systems require two gluteal IM injections to achieve the required studied dose in clinical investigations, due to the absorption-limited kinetic behavior of the nanosuspensions.[22,23] In the clinical investigations of these two systems, a 4-week, lead-in evaluation of safety and tolerability of oral dosage forms, are required as these systems cannot be removed after administration. Questions still remain regarding the reproducibility of complex manufacturing, reproducibility and predictability of PK/PD, and versatility of ARV drug loading in these nanosuspension LAI's.[24]

In-situ forming implant (ISFI) delivery systems offer advantages over the aforementioned nanosuspension LAI's, most notably the ease of manufacturing and capability to load a wide range of drugs, including proteins and hydrophobic molecules, while demonstrating release profiles ranging from days to 12 months. [25-27] Administered subcutaneously (SQ), ISFI's offers less invasive placement and easy removal in the case of adverse events. These systems are

biodegradable thus not requiring surgical removal once the loaded drug has been released.[25-29] *In situ* forming devices were first devised by Dunn *et al* in 1990 and the PK/PD have been reproduced and predicted in a number of human and veterinarian products.[30-34]

This liquid, syringeable system employs a water insoluble, biodegradable copolymer dissolved in a biocompatible, water miscible, organic solvent, afterwards drug is added to form a solution or suspension.[35] Polymers such as, poly(d,l-lactide)/poly(d,l-lactic acid) [PLA], poly(d,l-lactide-co-glycolide)/poly(d,l-lactic acid-co-glycolic acid)[PLGA], and poly(d,l-lactide-co-_-caprolactone) [PLC] are often used, yet PLGA is most commonly used due to its historically safe use in humans. Once in the body, PLGA eventually undergoes hydrolysis of its ester linkages to form biocompatible lactic and glycolic acids that are then further metabolized.[36] A few solvents are commonly used in this system, such as, propylene glycol, acetone, dimethyl sulfoxide (DMSO), tetrahydrofuran, glycofurol, and *N*-methyl-2-pyrrolidone (NMP). NMP is most frequently utilized due to its solubilizing power and safety in vivo. The FDA has designated NMP as "generally regarded as safe" (GRAS) material.[37,38] Once injected into the body, the solvent dissipates and extracellular fluid floods the organic phase. This phase separation leaves behind a depot comprised of the drug, copolymer, and any other rate-controlling additive(s).[39,40]

Presently, two FDA approved products are applying this type of delivery system, Eligard® and Atridox®. Both products utilize the Atrigel® ISFI delivery system. Eligard® is a sustained-release injection, formulated with PLGA, NMP and leuprolide acetate for the treatment of advanced prostate cancer.[41-45] Atridox® is another sustained-release injection, formulated with PLA, NMP and doxycycline hyclate, to treat periodontal disease of the sub-gingival space.[46,47] Another sustained-release injection formulated with bupivacaine in sucrose acetate isobutyrate (SAIB) and benzyl alcohol, Posimir®, is in a phase III clinical trial for the management of perioperative pain.[42]

Properties of the formulation, such as copolymer molecular weight, hydrophobicity, and ratio, as well as the solvent type, can be adjusted to achieve the desired drug release profile.[38] Some of the challenges that arise while pursuing an optimal formulation and drug release profile are improving drug loading and minimizing burst release of drug that occurs during the lag time between SQ administration and formation of the implant. Drug may reach a high peak plasma concentration during this lag time, which may result in systemic toxicity and ultimately sub optimal dosing frequency due to large drug loss initially post administration.[48,49]

To date, our laboratory has successfully formulated MK-2048 (Merck & Co), a novel second generation integrase strand transfer inhibitor (INSTI) in an ISFI system. In pharmacokinetic studies, this formulation, injected SQ, demonstrated a steady release of 15 mg MK-2048 for up to one year in NOD/Scid Gamma (NSG) and Bone/Liver/Thalamus (BLT) humanized mouse models. Our laboratory has also successfully formulated rilpivirine and dolutegravir (DTG, Trivicay, GlaxoSmithKline/ViiV Healthcare) separately, and both formulations demonstrated sustained drug release in plasma for up to 150 and 60 days respectively. All three of these formulations demonstrated potential effectiveness is humans as mean plasma drug concentrations were sustained above four times the concentration needed to inhibit 90% of viral replication (IC90). Along with these formulated ARVs, 18 other FDA-approved antiretroviral drugs have been screened and have solubility in NMP ranging from >20-700 mg/mL (avg. of >225 mg/mL). These ARVs also demonstrate potential in being formulated within the ISFI delivery system.

The pharmacokinetic profile of the three aforementioned formulations were then investigated in the rhesus macaque model, in collaboration with Dr. Garcia-Lerma (CDC), from these studies,

only dolutegravir demonstrated mean plasma concentrations that approached the IC90 of dolutegravir, 64 ng/mL. As a dolutegravir-based ISFI formulation provided significant protection from two high-dose HIV-1 challenges, six weeks apart, in BLT humanized mice, this formulation proves to be the most promising formulation to optimize and further develop (Benhabbour *et al*, manuscript in preparation). The aim of this study was to develop and characterize an ISFI formulation with increased dolutegravir loading to reach the efficacy threshold of mean plasma concentrations greater than 4x the PA-IC90 of dolutegravir. The overall goal was to optimize a formulation that would achieve a concentration of 300mg/mL in 2mL for a single injection that provides HIV-1 protection for at least three months. By optimizing the organic solvent system within the existing ISFI formulation using biodegradable/non – toxic solvents, we will achieve increased dolutegravir loading within the formulation. Here we present the characterization and *in vitro* and *in vivo* evaluation of the safety and efficacy of the optimized dolutegravir – based ISFI formulation.

2. Materials and Methods:

2.1 Materials

Dolutegravir was purchased from Shellekchem (Houston, TX, USA; Cat. No. S2667) and 50:50 poly(DL-lactide-*co*-glycolide) (PLGA) was purchased from LACTEL (Birmingham, AL, USA; Cat. No. B6010-1P, MW 27 kDa, inherent viscocity range 0.26-0.54 dL/g). *N*-methyl-2-pyrrolidone (NMP, <USP>) was purchased from ASHLAND (Wilmington, DE, USA; Product Code 851263, 100% NMP). Gelucire® 44/14 (Lauroyl polyoxyl-32 glycerides NF or Lauroyl Polyoxylglycerides) was purchased from Gattefosse (Saint Priest Cedex, France; Product Code 3051TPD), and the <USP> grade was purchased from Sigma Aldrich (Rockville, MD, USA; Cat. No 1356950). High Performance Liquid Chromatography (HPLC) grade Acetonitrile (ACN), Trifluoroacetic acid (TFA), Solutol-HS 15, and Phosphate Buffered Saline (0.01M PBS, pH 7.4) were all purchased from Sigma-Aldrich (St. Louis, MO, USA; ACN- Product Code 1002377876/Product No. 34998, TFA- Product Code 1002181743/Product No. T6508, Solutol-HS - Product Code 101822541/Product No. 42966, PBS-Product Code 1002006600/ Product No. P5368). Triacetin was purchased from Acros Organics part of Thermo Fischer Scientific (Geel, Belgium; Cat. Code 139220010). Polyethylene glycol 400 (PEG400) was purchased from EMD Millipore (Billerica, MA, USA; Cat. No. PX1286B-2). Puradisc™ 13mm syringe filter/0.2µm Nylon filter media was purchased from GE Healthcare Life Sciences (Cat. No. 6786-1302).

2.2 Experimental Animals

Balb(c) mice

2.3 Methods

2.2.1 *Dolutegravir Solubility Studies*

The solubility of DTG was investigated in various organic solvents and solvent ratios. Solvents were selected based on their biocompatibility and physiochemical properties of dolutegravir. All solvents chosen are in the FDA's Generally Recognized as Safe (GRAS) Substances (SCOGS) database, on the FDA's Inactive Ingredients List, meet USP/NF criteria, and have LD50's that would make them suitable for incorporation into ISFI systems.

Each solvent system was added in ~10 mg increments to ~20 mg of dolutegravir until drug was dissolved. Respective solutions were visually inspected after 0.5 h to ensure dolutegravir did not precipitate of solution. Another ~10mg increment was added if dolutegravir precipitated out of solution upon inspection. Aliquots of final solutions, along with appropriate dilutions, were taken

for analysis dolutegravir concentrations by HPLC methods (n=3). The results of this solubility study were used to determine the optimal solvent system used in the preparation of ISFI formulations.

2.2.2 Determination of dolutegravir concentration by High Performance Liquid Chromatography (HPLC)

Concentrations of dolutegravir from subsequent formulations and evaluations were determined via HPLC. The HPLC analysis was carried out with a Finnigan Surveyor HPLC system (Thermo Finnigan, San Jose, CA, USA) with a Photodiode Array (PDAD) Plus Detector, auto-sampler, and LC Pump Plus. The stationary phase utilized for the analysis was an Inertsil ODS-3 column (5 µm, 4.6 Å~ 150 mm, [GL Sciences, Torrance, CA, USA]) maintained at 40°C. The mobile phase used was composed of 0.1% trifluoroacetic acid (TFA) in 5% ACN- 95% double distilled water. The flow rate was 1.0 mL/min and effluents were measured at a wavelength on 265 nm. Sample solution was injected at a volume of 25µl. A calibration curve was prepared in the dolutegravir concentration range of 0.122–250 µg/ml (r = 0.9997-0.9999).

2.2.3 ***Preparation of dolutegravir ISFI formulations***

All formulations were prepared containing various concentrations of dolutegravir in various polymer to optimized solvent system ratios, by weight. Appropriate amounts of PLGA 50:50 were dissolved in either NMP alone or the optimized NMP co-solvent system at various ratios. Then the desired amount of dolutegravir was dissolved in the placebo solution for each evaluated formulation. Solution were then heated and vortexed to assist with drug dissolution in the formulation.

2.2.4 ***Saturation concentration of dolutegravir in improved placebo ISFI formulations***

Formulation solutions in PLGA and NMP/Gelucire (9:1) were prepared with 1:4, 1:6, and 1:8 ratios of PLGA to (NMP/Gel 9:1) along with a 1:4 PLGA: NMP (no Gel) formulation as a comparator. Each placebo ISFI formulation was prepared as described in 2.2.3. Each formulation solution was added in ~10 mg increments to ~10 mg of dolutegravir until drug was dissolved. Respective solutions were visually inspected after 0.5 h to ensure dolutegravir did not precipitate. Another ~10mg increment was added if dolutegravir precipitated out of solution upon inspection. Aliquots of final solutions, along with appropriate dilutions, were taken for analysis of dolutegravir concentrations via the HPLC method described in section 2.2.2 (n=3). The maximum concentrations of dolutegravir in each formulation solution are reported.

2.2.5 ***Stability***

The stability of the optimized 1:4 PLGA: (NMP/Gel 9:1) and 1:4 PLGA: NMP formulations were investigated under accelerated conditions of temperature and humidity (25°C ± 1°C, 60% RH; 40°C ±1°C, 75% RH). Time zero samples were used as controls. Samples were removed from chambers, at predetermined time points, (2, 4, and 7 days, then weekly thereafter), and were visually inspected for any deviations in color, odor, consistency, *etc*. from the time zero sample observation and for any drug precipitates. At each time-point, mentioned above, samples from each formulation were taken and analyzed for dolutegravir concentrations, via HPLC method described in section 2.2.2 (n=3). Sample dolutegravir concentrations were compared to time zero concentrations to calculate the amount of doluteravir loss.

2.2.6 ***Viscosity***

The viscosity of optimized dolutegravir ISFI formulations, of varying PLGA: (NMP/Gel 9:1) ratios were measured by a rheometer (Brookstone Digital Rheometer, Model CV-III V3. X RV Standalone). The temperature was maintained at 25 $\pm$ 2°C by a circulating water bath. A CP-40

cone spindle was utilized. 0.6 mL samples of each formulation were expelled into the sample cup and readings were taken at a motor speed of 1.5 rpm (n=3).

2.2.7 *Density*
The density of the optimized dolutegravir ISFI formulations of varying PLGA: (NMP/Gel 9:1) ratios, used in the *in vitro* release study, were calculated by determining the weight of 1 mL of each formulation held in a 1mL volumetric flask.

2.2.8 *Differential scanning calorimetry* (DSC)
Dolutegravir, as received, neat PLGA, a placebo optimized ISFI formulation, and a dolutegravir-loaded optimized ISFI formulation were analyzed by DSC. ISFI solutions were introduced, into 200mL of 2% Kolliphor/Solutol HS, 0.01M PBS at pH 7.4/37°C. The formulations remained in this media long enough for the implant to fully form (24-48hrs), after which point implants were allowed to air dry (3-7 days). Samples varying in weight from 7-10mg were weighed and then hermetically sealed in an aluminum pan and placed in the differential scanning calorimeter. The samples were heated from 25 – 250 °C, at a heating rate of 10°C/min, under nitrogen atmosphere (flow rate 20 mL/min). The thermograms were used to determine the peak glass transition temperature (T_g) of PLGA and the peak transition temperature of dolutegravir. The transition temperatures of and dolutegravir in ISFI formulations were compared to neat PLGA and pure dolutegravir.

2.2.9 *Scanning Electron Microscopy (SEM) Imaging*
Implant surface and microstructures were evaluated by scanning electron microscopy (SEM). Implants were first prepared by injecting 20 µL of polymer solution into 10 mL of 0.1 M PBS, pH 7.4 at 37°C. At predetermined time points (1, 3, 7, 14, and 30 days post injection), the implants were removed from the bath solution, flash-frozen, and then fractured over dry ice. Following freeze-fracture, implants were lyophilized for 24 h (SP VirTis Advantage XL -70, Warminster, PA, USA). The lyophilized samples were then mounted on an aluminum stub using carbon tape, and sputter coated with 5 nm of gold-palladium alloy (60:40) (Hummer X Sputter Coater, Anatech USA, Union City, CA, USA). The coated samples were then imaged using a Zeiss Supra 25 field emission scanning electron microscope with an acceleration voltage of 5kV, 30µm aperture, and average working distance of 10mm (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA).

2.2.10 *In vitro release study of dolutegravir from optimized dolutegravir ISFI* formulations
Optimized dolutegravir ISFI formulations of varying PLGA: (NMP/Gel 9:1) ratios were introduced, into 200mL of 2% Kolliphor/Solutol HS, 0.01M PBS, pH 7.4 at 37°C. 1 mL aliquots were collected at predetermined time points (n=3) and replaced with 1mL of fresh media. Samples were analyzed by HPLC as described in section 2.2.2, to determine the concentration of dolutegravir. The percent dolutegravir release was plotted overtime for each formulation.

2.2.11 *In vivo* safety and pharmacokinetic study of optimized dolutegravir ISFI formulations
Female balb (c) mice, 6 to 8 weeks (Jackson Laboratory), were housed in a pathogen-free room. All experiments involving the mice were carried out with an approved protocol by the University of North Carolina Animal Care and Use Committee.

Animals were randomly divided into two groups (N=7): group A and group B. Mice were assigned to receive a single 250 mg/kg dose of optimized dolutegravir ISFI formulations administered subcutaneously. Group A mice were injected with formulation A (1.5:1:4 w/w/w (DTG: PLGA: (NMP/Gel 9:1) [DTG]=210mg/mL) and group B mice were injected with

formulation B (0.6:1:2 w/w/w (DTG: PLGA: (NMP/Gel 9:1) [DTG]=142mg/mL). Formulation B was evaluated as a comparator to the previous formulation containing NMP alone. Peripheral blood samples were collected longitudinally at specific time points (days 3, 7, 14, 30 and 60) into EDTA coated capillary tubes, to separate plasma for safety (proinflammatory cytokines, IL-6 and TNF-Alpha) and PK analysis. Plasma samples were analyzed using a validated liquid chromatography-tandem mass spectrometry (LCMS/MS) method in collaboration with Dr. Kashuba (UNC Pharmacology Core). Tissue samples from the female reproductive tract (FRT) were harvested at 7 days (n=3), 28days (n=6), and 84 days (n=2). All samples were stored at -80°C until PK analysis. Skin samples from the injection site were collected on days 3, 7, 14, 30 and 60 and analyzed for injection site reactions (H&E staining).

2.2.12 *Pharmacokinetic Data Analysis*

Initial estimates for PK parameters were obtained through noncompartmental methods using WinNonlin Phoenix 6.1 (Pharsight, Mountain View, CA) on the composite median PK profile. To estimate PK parameters, a composite approach was deployed as previously described by Cottrell *et al*.[50] The terminal elimination rate constant ($K_{el,\beta}$) was estimated by linear regression of the terminal portion of the log-transformed concentration versus time curve using at least the last three data points in the terminal elimination phase. The maximum observed concentration (Cmax) was obtained by observation from the concentration-time profile. The area under the curve (AUC) from time 0 to the last measured concentration was determined using the trapezoidal rule with linear-up/log-down interpolation. Data analysis, formatting, and exploratory plots were performed with R as previously reported by Cottrell *et al.*[50]

3. Results and Discussion

3.1 *Solubility study of dolutegravir*

The solubility of dolutegravir was determined in triacetin, PEG 400, and Gelucire 44/14 along with varying combinations of these organic solvents with NMP (Table 1). Dolutegravir is poorly soluble or insoluble in ethanol and water. Shelleckchem reports good solubility of dolutegravir in dimethyl sulfoxide (DMSO), yet DMSO is not a suitable solvent due to its toxicity and skin irritating properties.[51] Solvents were selected due to their polar aprotic properties and/or non-toxic profile. The solubility of dolutegravir in PEG 400 and triacetin was considerably decreased compared to NMP; solvent systems of PEG400/NMP and triacetin/NMP also demonstrated lower solubility of dolutegravir than NMP alone. Gelucire, on the other hand, modestly increased the solubility of dolutegravir compared to NMP. The solvent system of NMP/Gel (9:1) demonstrated the greatest increase in dolutegravir concentration (w/w) from NMP at 0.26546 ± 0.0016.

Table 1: Solubility of dolutegravir in prospective solvents and cosolvent systems

Solvent(s)	dolutegravir Concentration (w/w) (normalized to 1mg) (n=3) ± SD
NMP	0.16385 ± 0.0015
NMP/Gelucire 90/10	0.26546 ± 0.0016
NMP/Gelucire 80/20	0.23757 ± 0.0049
NMP/Gelucire 50/50	0.19011 ± 0.0022
PEG 400	0.01039 ± 0.0002
NMP/PEG 400 90/10	0.15649 ± 0.0004
NMP/PEG 400 80/20	0.14874 ± 0.0005
NMP/PEG 400 50/50	0.12245 ± 0.0053
Triacetin	0.08218 ± 0.0044
NMP/Triacetin 90/10	0.16203 ± 0.0020
NMP/Triacetin 80/20	0.15115 ± 0.0022
NMP/Triacetin 50/50	0.08291 ± 0.0007

Gelucires are non-ionic water-dispersible surfactants composed of mono-, di- and triglycerides and mono- and diesters of PEG. The two numbers following each Gelucire is specific to the characteristics of each one. The first number indicates the melting point and the second number indicated the Hydrophillic-Lipophillic Balance (HLB) value. Specifically, Gelucire 44/14 has a melting point of 44°C and HLB value of 14. The inertness of Gelucire, along with its solubilization capacity and low melting point, make it an appropriate co-solvent to aid in the solubilization of poorly-water soluble active pharmaceutical ingredients (APIs).[52]

While the addition of Gelucire improved the solubility of dolutegravir in each ratio of NMP/Gel tested, a trend of increasing dolutegravir solubility with decreasing Gelucire was observed. The effects of various co-solvents on the mechanism of solubilization of Gelucire has not been fully elucidated. Kawakami K, Miyoshi K, and Ida Y, found that while the addition of DMSO or dimethylacetoamide (DMA), to an aqueous solution of Gelucire, increased the solubility of poorly water-soluble model drugs, indomethacin and phenytoin, DMSO and DMA both increased the critical micelle concentration (CMC) of Gelucire and impacted the micellar characteristics. DMSO and DMA affected the micelle morphology in different ways. Whereas DMA increased, DMSO decreased the size and/or aggregation of micelles.[53] To our knowledge, no such investigations have been carried out utilizing NMP as a cosolvent, or Gelucire in a non-aqueous solution. It is hypothesized that some physiochemical interactions are at play that would expound on the solubility trend observed with varying concentrations of NMP and Gelucire. On the other hand, we hypothesized that it is more likely that at higher concentrations Gelucire simply transitions from acting as a solubility enhancer to a solute, competing with dolutegravir for solubilization in NMP. As the co-solvent system of NMP/Gel 9:1 demonstrated the greatest solubilization capacity of dolutegravir, this co-solvent system was utilized in the ISFI formulations further studied and discussed.

3.2 *Maximum concentration of dolutegravir in improved placebo ISFI formulations*

The maximum concentration of dolutegravir in the improved placebo ISFI formulations, along with a formulation composed of PLGA and NMP only as a comparator, was determined (Table 2). Ratios of PLGA: (NMP/Gel 9:1) were chosen as these ratios were hypothesized to most likely demonstrate a release of dolutegravir for at least three months given evidence from prior studies (Benhabbour *et al*, manuscript in preparation).

The maximum concentration of dolutegravir in all three improved ISFI formulations were similar with the 1:8 PLGA: (NMP/Gel 9:1) formulation allowing for the greatest dolutegravir loading, with a concentration (w/w) of 0.16194 $\pm$ 0.0113. All three ISFI formulations incorporating the improved co-solvent system demonstrated greater maximum concentrations of dolutegravir versus the 1:4 PLGA: NMP comparator formulation. The observed increase in dolutegravir loading capacity within the ISFI formulation was to a lesser degree than the increase in solubility in the solvent system alone, as expected.

Table 2: Maximum concentration of dolutegravir in improved ISFI formulations

ISFI Formulation	dolutegravir Concentration (w/w) (normalized to 1mg) (n=3) $\pm$ SD
1:4 PLGA:NMP	0.12293 $\pm$ 0.0007
1:4 PLGA: (NMP/Gel 9:1)	0.15825 $\pm$ 0.0015
1:6 PLGA: (NMP/Gel 9:1)	0.15158 $\pm$ 0.0020
1:8 PLGA: (NMP/Gel 9:1)	0.16194 $\pm$ 0.0113

3.3 *Stability*

Table 3: Investigation of dolutegravir ISFI formulations stability under accelerated conditions

Time (days)	Condition	Residual Dolutegravir (%) 0.8:1:4 DTG: PLGA: NMP (16.3% DTG w/w)	Residual Dolutegravir (%) 1.1:1:4 DTG: PLGA: (NMP/Gel 9:1) (22.4% DTG w/w)
0	25°C/60% RH	100.0%	100.0%
	40°C/75% RH	100.0%	100.0%
2	25°C/60% RH	95.8%	98.3%
	40°C/75% RH	95.1%	104.4%
4	25°C/60% RH	93.2%	106.1%
	40°C/75% RH	95.4%	117.1%
7	25°C/60% RH	91.4%	98.9%
	40°C/75% RH	96.5%	97.6%
14	25°C/60% RH	88.4%	
	40°C/75% RH	79.3%	

The physical appearance, odor, and consistency were noted along with the concentration of dolutegravir analyzed via HPLC for 1.1:1:4 DTG: PLGA: (NMP/Gel 9:1) and 0.8:1:4 DTG: PLGA: NMP ISFI formulations, stored at, 25°C ± 1°C, 60% RH and 40°C ±1°C, 75% RH, at 0, 2, 4, 7, and 14 days (n=3). The residual dolutegravir percentage from each formulation are reported (Table 3). There was no change in the physical appearance from 0-7 days for the NMP only comparator formulation, yet there was a change in the appearance and consistency in the 1.1:1:4 DTG: PLGA: (NMP/Gel 9:1) formulation. At one week amounts of Gelucire precipitated out solution and adhered to the walls of the vial. This finding was more pronounced in the 1.1:1:4 DTG: PLGA: (NMP/Gel 9:1) formulation stored at 25°C ± 1°C, 60% RH. This was as expected given the melting temp of Gelucire is 44°C. Both formulations appeared to have various components completely out of solution and layered in their respective vials at 14 days. As seen in Table 3, the residual dolutegravir percentage in the 0.8:1:4 DTG: PLGA: NMP formulation decreased from 100% to 88.4% at 25°C ± 1°C, 60% RH and 79.3% at 40°C ± 1°C, 75% RH in 14 days. The residual dolutegravir percentage in the 1.1:1:4 DTG: PLGA: (NMP/Gel 9:1) decreased from 100% to 98.9% at 25°C ± 1°C, 60% RH and 97.6% at 40°C ± 1°C, 75% RH in 7 days. While the decrease in residual dolutegravir was much larger for the NMP only formulation, the decrease in the residual dolutegravir percentage of the improved formultation was less than 4% under any condition and at any duration of storage up to 7 days. Gelucire may be playing a role in minimizing the amount of dolutegravir lost over time. The limiting aspect for the stability of this formulation is Gelucire remaining in solution. These results indicate that the improved formulation is stable with negligible dolutegravir lost and minimal amounts of Gelucire precipitating out of solution at 7 days.

3.4 *Viscosity*

To ensure that the increase in dolutegravir concentration, addition of Gelucire, or varying PLGA: (NMP/Gel 9:1) ratio did not alter the syringability of this formulation, the viscosity of placebo and dolutegravir loaded improved formulations at varying PLGA: (NMP/Gel 9:1) was determined. The investigated ratios of PLGA: (NMP/Gel 9:1) were 1:4, 1:6, 1:8, and 1:24 along with a 1:4 PLGA: NMP formulation as a comparator (Table 4). The viscosity of placebo formulations ranged from 7.2 ± 0.2 to 98.1 ± 1.0 cP (centipoise) and dolutegravir loaded formulations ranged from 14.8 ± 0.3 to 187.3 ± 3.1 cP, at 25°C and 1.5 rpm. Both the viscosity of placebo and dolutegravir formulations decreased with the decrease in PLGA: (NMP/Gel 9:1) ratio. The

addition of Gelucire to ISFI formulations increased the viscosity of the formulation. The viscosity of 1:6 PLGA: NMP was 24.5 ± 0.6 cP compared to that of 1:6 PLGA: (NMP/Gel 9:1), which was 39.0 ± 0.6 cP. The viscosities of 1.3:1:4 DTG: PLGA: NMP and 1.3:1:4 DTG: PLGA: (NMP/Gel 9:1) were 48.3 ± 0.6 cP and 71.0 ± 1.3 cP. Notably, the addition of dolutegravir in all formulations increased the measured viscosity, almost 2-fold, compared to placebo formulations. The increases in viscosities, while significant, are not great enough to impact the syringability of ISFI systems through a 20-25G sized needle.

Table 4: Viscosity of ISFI formulations of varying PLGA: co-solvent ratios

Placebo ISFI Formulations					
	1:4 PLGA: (NMP/Gel 9:1)	1:6 PLGA: (NMP/Gel 9:1)	1:8 PLGA: (NMP/Gel 9:1)	1:24 PLGA: (NMP/Gel 9:1)	1:6 PLGA: NMP
Viscosity (cP) ± SD	98.1 ± 1.0	39.0 ± 0.6	17.3 ± 0.5	7.2 ± 0.2	24.5 ± 0.6
dolutegravir ISFI formulations					
	0.9:1:4 DTG: PLGA: (NMP/Gel 9:1) (18.9% DTG w/w)	1.3:1:6 DTG: PLGA: (NMP/Gel 9:1) (19.0% DTG w/w)	1.7:1:8 DTG:PLGA: (NMP/Gel 9:1) (18.7% DTG w/w)	4.8:1:24 DTG: PLGA: (NMP/Gel 9:1) (19.0% DTG w/w)	1.34:1:6 DTG:PLGA:NMP (19.2% DTG w/w)
Viscosity (cP) ± SD	187.3 ± 3.1	71.0 ± 1.3	41.0 ± 1.3	14.8 ± 0.3	48.3 ± 0.6

3.5 *Density*

The densities of placebo formulations with various ratios of PLGA: (NMP/Gel 9:1), 1:4, 1:6, 1:8, 1:10, and 1:24, were determined (Table 5). Densities ranged from 1.028 to 1.057 g/mL and decreased with decreasing ratio of PLGA: co-solvent system as expected.

Table 5: Density of ISFI formulations of varying PLGA: co-solvent ratios

	1:4 PLGA: (NMP/Gel 9:1)	1:6 PLGA: (NMP/Gel 9:1)	1:8 PLGA: (NMP/Gel 9:1)	1:10 PLGA: (NMP/Gel 9:1)	1:24 PLGA: (NMP/Gel 9:1)
Density (g/mL)	1.057	1.047	1.045	1.039	1.028

3.6 *DSC*

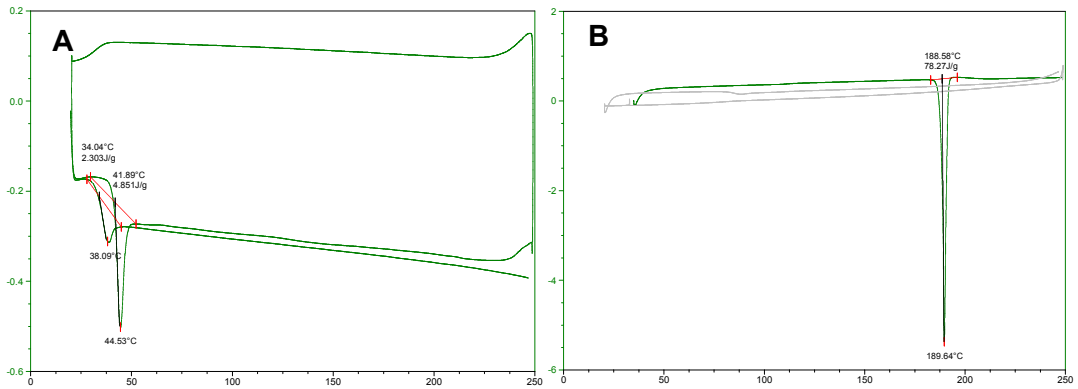

Figure 1: DSC thermograms of 50:50 PLGA as received (A) and neat dolutegravir (B).

DSC thermograms of 50:50 PLGA as received and neat dolutegravir, are illustrated in (Figure 1: A-B). The Amorphous nature of PLGA is indicated by the broad endothermic peak at 44.53°C. This analysis confirmed the Tg of PLGA which is approximately 45-50°C. Pure dolutegravir produced a sharp endothermic peak at 189.64°C. Samples of placebo and dolutegra vir loaded 1:2 PLGA: (NMP/Gel 9:1) formulations are to be analyzed. Hopefully the results from these samples will indicate that no chemical interaction occurred between 50:50 PLGA and dolutegravir, that these components remained stable within the formulation, and that dolutegravir remained in its crystalline state in the depot.

3.7 *SEM Imaging*

SEM cross-sectional images of placebo and dolutegravir loaded ISFIs post incubation from in vitro release media (2% Kolliphor/Solutol HS, 0.01M PBS, pH 7.4 at 37 ºC), over 30 and 28 days respectively, are illustrated in (figures 2 and 3).

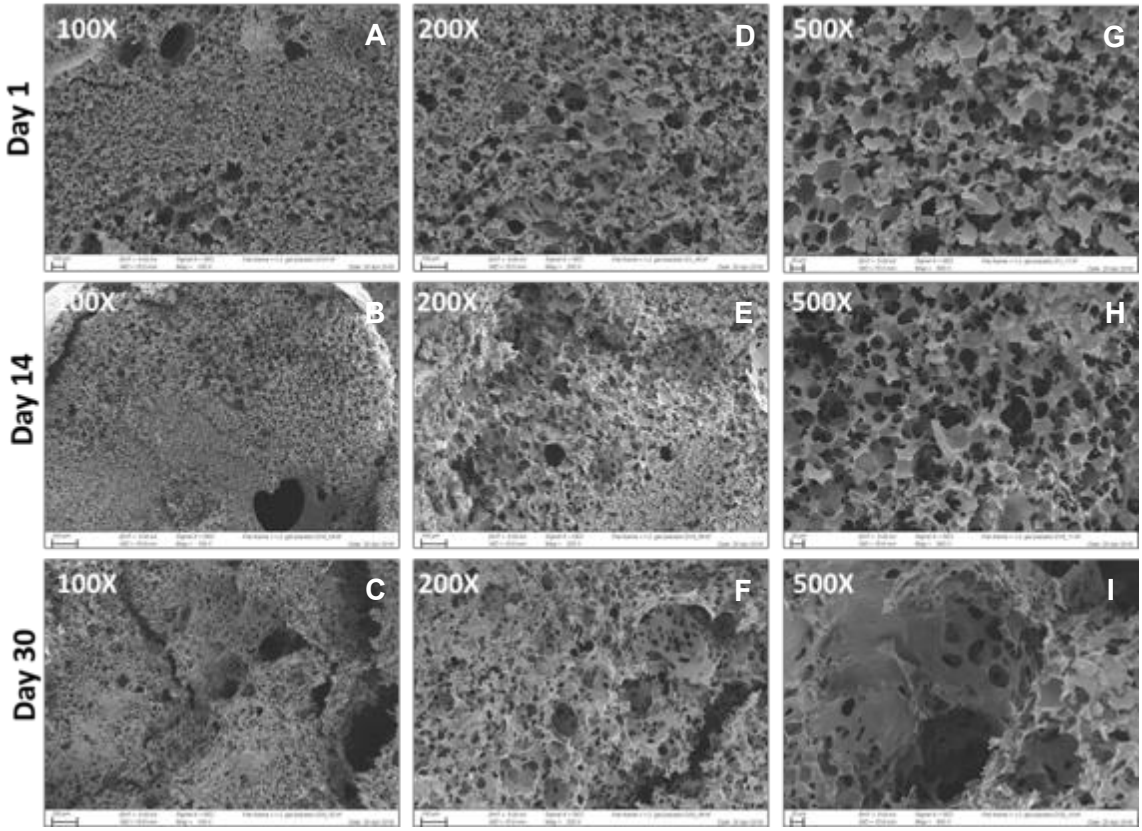

Figure 2: The SEM cross-section images of Placebo ISFIs (1:2 PLGA/(NMP/Gel), PLGA MW 27 KDa) over 30 days. Illustration of the effect of PLGA degradation over 30 days on the depot microenvironment. Column 1 is a low magnification image of the entire depot, column 2 is a higher magnification image of the core, and column 3 is a higher magnification image of the center of the depot. Rows 1-3 collection time points of implants post incubation in 2% Kolliphor/Solutol HS, 0.01M PBS, pH 7.4 at 37ºC. (A-C) 100x magnification, (D-F) 200x magnification, (G-I) 500x magnification.

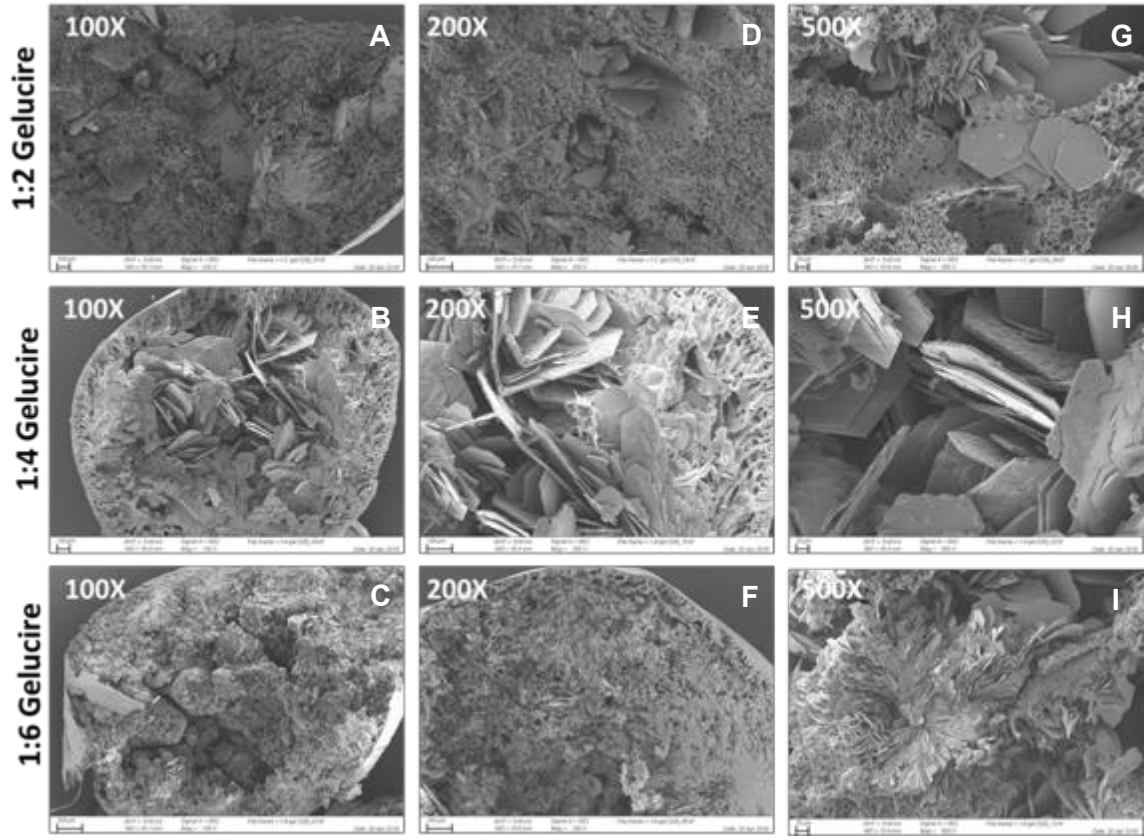

Figure 3. The SEM cross-section images of DTG ISFIs after drug release for 28 days. Illustratio of the effect of PLGA/NMP ratio on the depot microenvironment and its correlation to drug release kinetics at day 30. Each row is a representation of ISFIs containing a different ratio of PLGA to NMP. Column 1 is a low magnification image of the entire implant, Columns 2 and 3 are higher magnification images focusing on the center of the implant. A 1:2 PLGA/NMP 100x, B 1:4 PLGA/NMP 100x, C 1:6 PLGA/NMP 100x, D 1:2 PLGA/NMP 200x, E 1:4 PLGA/NMP 200x, F 1:6 PLGA/NMP 200x, G 1:2 PLGA/NMP 500x, H 1:4 PLGA/NMP 500x, I 1:6 PLGA/NMP 500x.

3.8 *In vitro release study of dolutegravir from ISFI formulations*

The dolutegravir release profile from ISFI formulations are depicted in (Figure 4). Both 5.8:1:24 DTG: PLGA: (NMP/Gel 9:1) and 2.6:1:8 DTG: PLGA: (NMP/Gel 9:1) released 100% of dolutegravir, with the 5.8:1:24 formulation doing so in the shortest amount of time. The 1.0:1:4 DTG: PLGA: (NMP/Gel 9:1) formulation released the smallest percentage of dolutegravir at 57.53%.

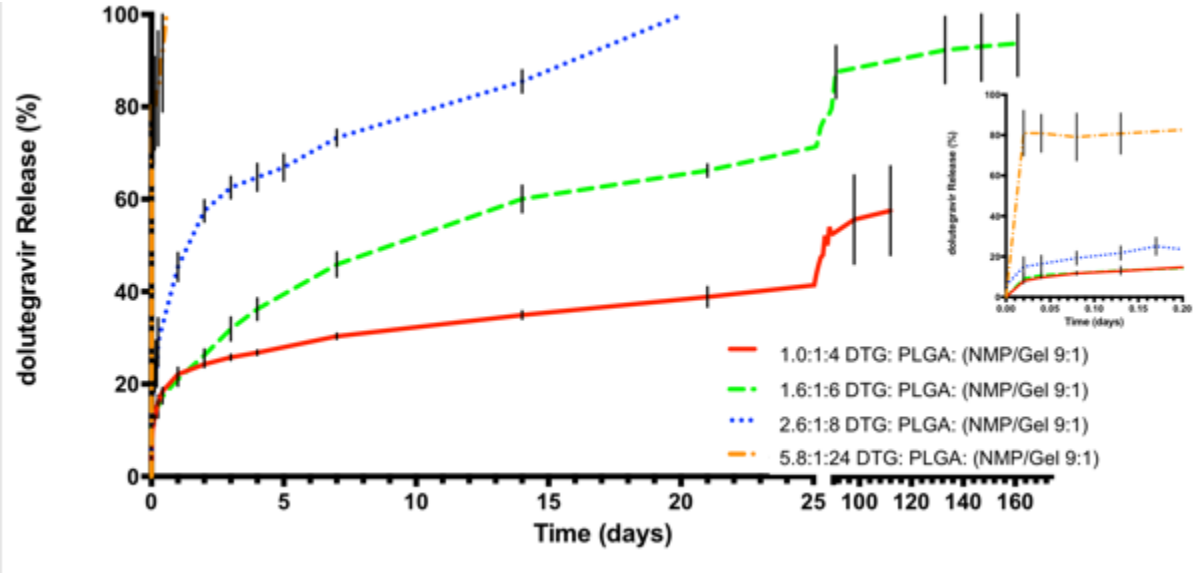

Figure 4: *in vitro* PK release profiles of optimized PLGA: (NMP/Gel 9:1) ISFI formulations. Illustration of the PK effect of varying PLGA: (NMP/Gel 9:1) ratios in ISFI formulations. Formulations were introduced, into 200mL of 2% Kolliphor/Solutol HS, 0.01M PBS at pH 7.4/37°C. Samples were taken at predetermined time points, as described on 2.2.10, and analyzed by HPLC, as described in 2.2.2.

The release profile of ISFI systems are characterized by two phases. The initial burst drug release phase, then a slowed, more sustained drug release phase. The initial burst release phase is a result of the fast diffusion of the dolutegravir during the lag time between the injection of formulation solution and formation of the solid implant. It is also the result of the rapid diffusion of the dolutegravir molecules entrapped on the outer most surface of the implant. The initial release burst occurred within the first 6 hours for all four formulations. The formulation with the largest PLGA: (NMP/Gel 9:1) ratio, 1:4, produced the smallest burst release of 16.01%. The formulation with the smallest PLGA: (NMP/Gel 9:1) ratio, 1:24, produced the largest burst release of 83.97%. Larger amounts of PLGA within the formulation increases the viscosity, as described in 3.4, as well as increases the interaction of dolutegravir with the forming polymer matrix. Both factors result in the diminished diffusion of dolutegravir from the formulation. The following sustained drug release phase is described by the continuous and moderate increase in the release of dolutegravir. The degree of the release is also dependent on the amount of PLGA in the formulation. The formulation with the smallest PLGA: (NMP/Gel 9:1) ratio, 1:24, released the remaining amount of dolutegravir within 24 hrs, while the 1:4 PLGA: (NMP/Gel 9:1) formulation still had a remaining 42.47% of dolutegravir within the implant at the last measured time point of 121 days. The increased concentrations of PLGA significantly decrease the rate of drug release from ISFI systems. While the 1:6 PLGA: (NMP/Gel 9:1) formulation ratio provided the greatest overall dolutegravir percentage of release, it was determined that this formulation would result in an NMP amount nearing the FDA's limit of NMP injected during a single 2 mL SQ injection. Thus the 1:4 PLGA: (NMP/Gel 9:1) was identified as the optimal ratio producing zero-order or prolonged release for 3 months after injection into aqueous media.

3.9 *In vivo* safety and pharmacokinetic study of optimized dolutegravir ISFI formulations
The in vivo plasma concentration profiles from treatment group A (formulation A - 1.5:1:4 w/w/w (DTG: PLGA: (NMP/Gel 9:1) [DTG]=210mg/mL) and group B (formulation B - 0.6:1:2 w/w/w (DTG: PLGA: (NMP/Gel 9:1) [DTG]=142mg/mL) are depicted in (Figure 5A). Formulation B was evaluated here as a comparator to the original ISFI formulation, previously studied, containing NMP alone (0.4:1:2 w/w/w (DTG: PLGA: (NMP/Gel 9:1) [DTG]=100mg/mL). Plasma concentrations were quantitated using a validated high-performance liquid chromatography-mass spectrometry LC/MS-MS method[50] and are plotted over time in (Figure 5). Noncompartmental analyses (NCA) of the median composite PK profiles demonstrated a bi-exponential decay for all three DTG ISFI formulations. After an initial first-order decline in plasma concentrations, DTG release approached zero-order kinetics (Figure 5B).

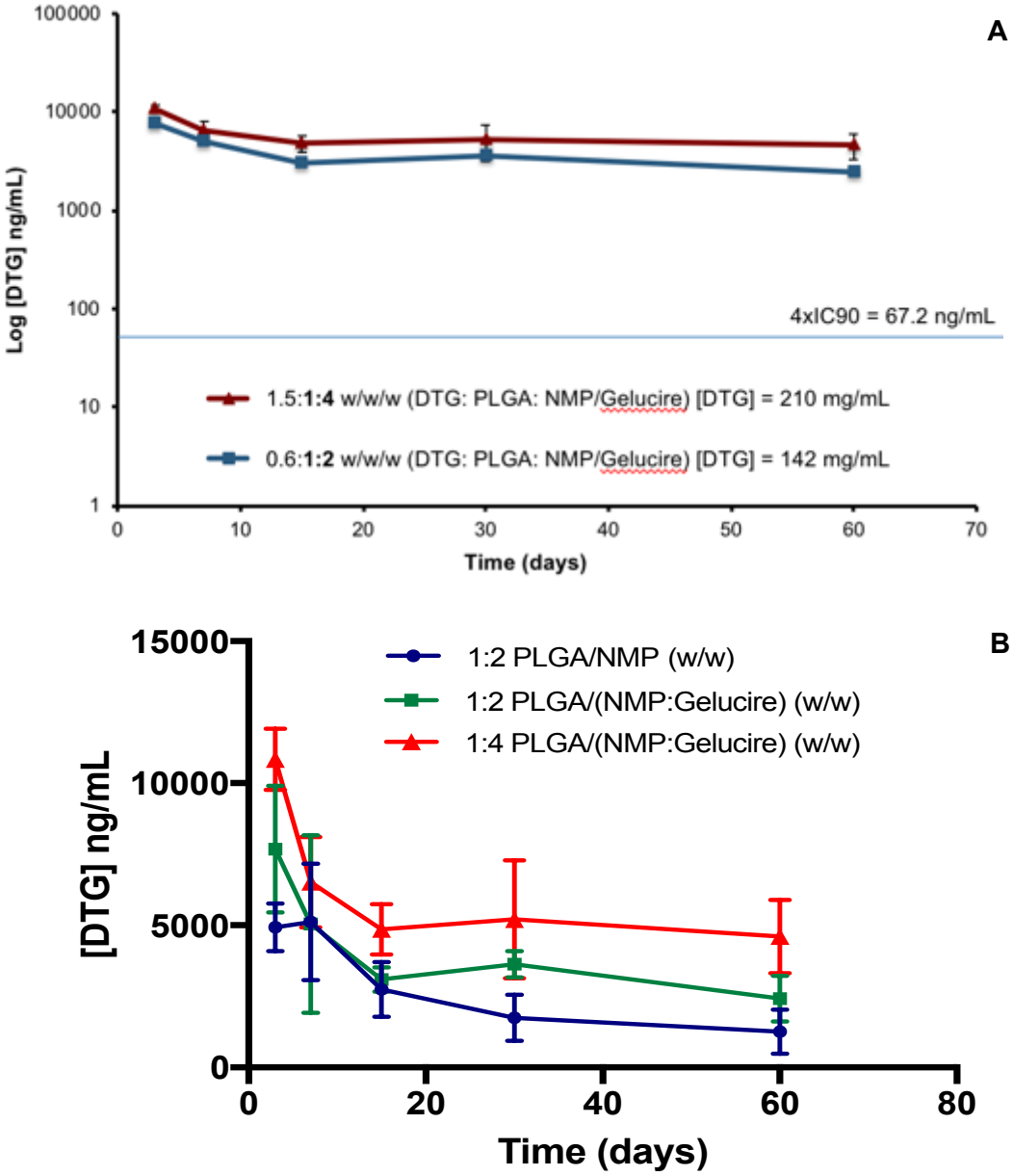

Figure 5: Comparison of plasma concentration of DTG produced from an original ISFI (PLGA/ NMP) and optimized ISFI (PLGA/(NMP/Gel 9:1)). Mice (N=6 per group) were injected with placebo or DTG-loaded optimized ISFI (PLGA/(NMP/Gel)) and plasma samples were collected

over 60 days and analyzed for DTG in collaboration with Dr. Kashuba and the UNC-CFAR Pharmacology Core, as described in 2.2.11 and 2.2.12. The PK data showed that plasma DTG levels were sustained above 4x IC90 of DTG over the course of the study (A) and were 3.86-fold and 5.55-fold day 30 and 60 than the original ISFI formulation (PLGA/NMP) at day 30 and 60, respectively.

Plasma concentrations of dolutegravir produced by formulation A and formulation B from the in vivo pharmacokinetic study were well above 4x the IC90 (67.2 ng/mL) of dolutegravir during the full duration of the study (Figure 5A). These results were expected as the original formulation containing NMP as the only solvent, with a lower concentration of dolutegravir, also produced plasma concentrations that were above 4x IC90 in a previous *in vivo* pharmacokinetic study (Benhabbour *et al*, manuscript in preparation). The produced plasma concentrations from formulations A and B along with the original ISFI formulation containing NMP alone are compared in (Table 6) (Benhabbour *et al*, manuscript in preparation).
At 7 days post-injection, the average dolutegravir plasma concentration produced by formulations A and B were 6,518.33 ng/mL and 5,056.66 ng/mL, respectively. These concentrations were 2.68x and 3.46x greater than the average concentration produced by the original ISFI formulation – 1,880 ng/mL. Compared to the average concentration produced by the original ISFI formulation at 30 days post-injection, 938,ng/mL, formulations A and B produced concentrations of 5,208.33 ng/mL and 3,628.33 ng/mL respectively. The average dolutegravir plasma concentration produced by formulation A was 5.55x that of the original ISFI formulation at this time point.

These results demonstrate the efficacy of the co-solvent in enhancing the solubility of DTG in the ISFI formulation and the ability of the optimized formulation to achieve higher plasma concentrations that far exceed the levels that were obtained with the original formulation.

Table 6: *In vivo* average dolutegravir plasma concentrations in ISFI injected Balb(c) mice

	Time (days)				
Formulation	3	7	15	30	60
1.5:**1:4** DTG:PLGA: (NMP/Gel 9:1) **[DTG] = 210 mg/mL**	10,838.33 ng/mL	6,518.33 ng/mL	4,851.66 ng/mL	5,208.33 ng/mL	4,605.0 ng/mL
0.6:**1:2** DTG:PLGA: (NMP/Gel 9:1) **[DTG] = 142mg/mL**	7,676.66 ng/mL	5,046.66 ng/mL	3,088.33 ng/mL	3,628.33 ng/mL	2,420.0 ng/mL
0.42:**1:2** DTG:PLGA:NMP **[DTG] = 100 mg/mL**		1,880 ng/mL		938 ng/mL	

Safety studies showed that both placebo and DTG-loaded PLGA/(NMP/Gel) formulations have low to no inflammation at the injection site (H&E staining, Figure 6). In these studies, there were 4 mouse groups (BALB/c, N=5 per group): Group A, formulation A - 1.5:1:4 w/w/w (DTG: PLGA: (NMP/Gel 9:1) [DTG]=210mg/mL); Group B, formulation B - 0.6:1:2 w/w/w (DTG: PLGA: (NMP/Gel 9:1) [DTG]=142mg/mL); Group C, 1:2 PLGA:(NMP/Gel) Placebo; and Group D, Saline control group. Skin samples were collected at predetermined time points (day 3, 7, 14, 30 and 60) and analyzed for injection site reactions (H&E staining). As shown in Figure 6, a very small amount of macrophages with add-mixed lymphocytes and plasma cells surrounded the

depot formulation in the early time point (day 3). At later time points, no macrophages or lymphocytes were present indicating that the depot was very well tolerated and the presence of macrophages and lymphocytes at the early time point was likely associated with inflammation from the injection.

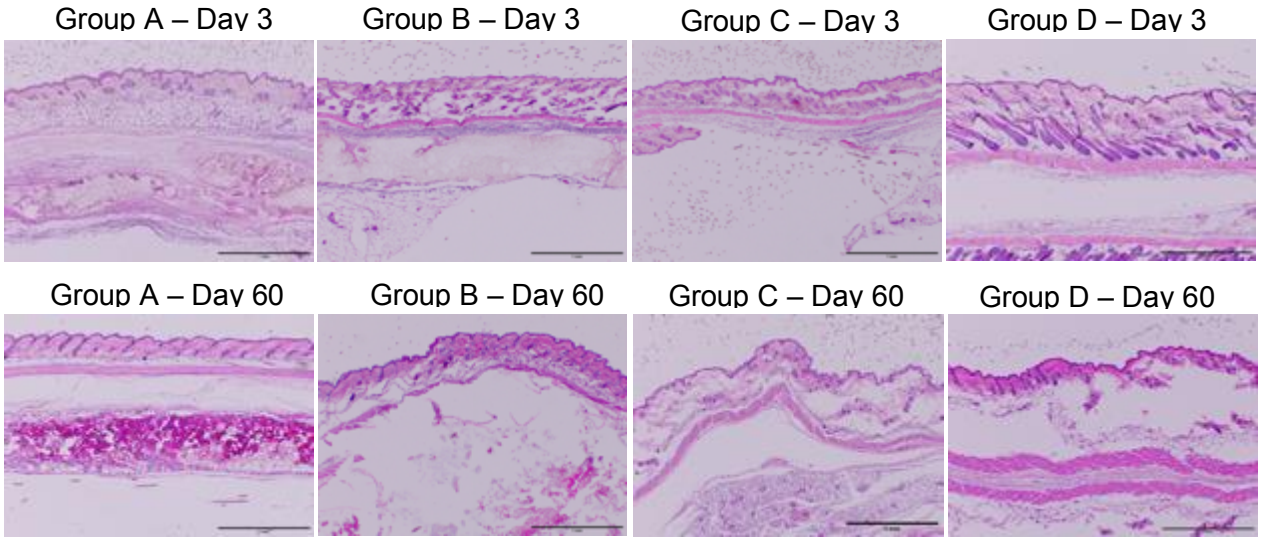

Figure 6: Representative micrographs of H&E-stained mouse subcutaneous tissue from the injection site after 3 days or 2 months post-administration of DTG ISFIs, placebo ISFI, or saline control.

Systemic inflammation was also evaluated using ELISA and testing for levels of pro-inflammatory cytokines (INF-α and IL-6) in plasma samples that were collected at predetermined time points (day 3, 7, 15, and 30). Results showed no detectable systemic inflammation (negative for both INF-α and IL-6 by ELISA, data not included) for all ISFI formulations at all time points.

These results indicate that both formulations A and B meet the understood HIV prevention efficacy threshold of producing ARV plasma concentrations that are maintained well above 4x the IC90 for that ARV in this balb(c) model. Formulations A and B were also very well tolerated in this model. As expected the increase in drug-loading capacity of formulation B, and more so with formulation A, resulted in dolutegravir plasma concentrations significantly greater than that produced by the original ISFI formulation.

4. Conclusion

HIV remains a global health crisis, and while there are current methods of PrEP in clinical development, ISFI systems offer advantages over these methods. There have been no R&D efforts made, to our knowledge, to adapt or modify an ISFI system for the delivery of long-acting antivirals. In this present study, we improved upon a dolutegravir ISFI formulation and proceeded with the characterization and *in vitro* and *in vivo* evaluation of such formulation. The improved formulation consisted of dolutegravir, 50:50 PLGA, and a co-solvent system of NMP/Gel 9:1, in a 1.5:1:4 ratio, respectively. The addition of the semi-solid, commercial solubilizing agent, Gelucire, increased the drug loading capacity of dolutegravir within the ISFI formulation by 1.28x. Kawakami K *et al*, note that the combination of a surfactant and co-solvent [DMSO] offered little advantage over either alone, yet this simple increase in dolutegravir

concentration was enough to result in plasma concentrations of dolutegravir above 4x IC90 of dolutegravir, and these concentrations far exceeded the levels that were obtained with the original formulation, in an in vivo pharmacokinetic study using balb(c) mice. The improved ISFI formulation provided a prolonged release of dolutegravir exceeding 3 months' time *in vitro*, 2 months in balb (c) mice without concern for tolerability of toxicity. The only concern regarding this formulation, is its stability given the semi-solid state of Gelucire at room temperature. To address this concern, the formulation could simply be briefly heated well above the melting point of Gelucire, and thoroughly vortexed, 1-2 hrs before planned administration. Along with this proposed, short-term solution, the storage and delivery of this formulation will be investigated with its further development. These results from an improved dolutegravir ISFI system support its use as a more appropriate alternative to current methods of PrEP in clinical development.

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